

## **A comparison in protein extraction from four major crop residues in Europe using chemical and enzymatic processes-a review**

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**A comparison in protein extraction from four major crop residues in Europe using  
chemical and enzymatic processes**

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**Highlights**

Separation of crop waste into different constituents containing higher protein contents

High protein yields using protein protocols relying on organic solvents, alkalis and acids

Proteases appear to be effective in recovering high protein yields

Lower yields with carbohydrases but functionality likely to be retained

Mechanical pre-treatments can increase protein yields during an enzymatic approach

**Abstract**

The agricultural production of olives, rapeseed, tomatoes and citrus fruits within Europe is significant, resulting in a considerable amount of residual material. Rapeseed contains a high proportion of protein but the presence of anti-nutritional components, including glucosinolates, limits its use in food and feed applications. In contrast, the protein quantities associated with the other crop residues are much lower, although each of the residues could be separated into different constitutive parts where some have shown higher protein contents. . A variety of different enzymatic based approaches to deconstruct crop residues have shown to be effective in increasing the yields of protein recovered. These studies show that valorisation of selected crop components could form the basis of a crop biorefinery process to capture proteins and other potentially useful compounds.

## **Keywords**

Protein; enzyme-assisted; olive; rapeseed; glucosinolate; Celluclast

## **1. General Introduction**

Food waste in the EU is estimated to be 38% of the whole crop yields with the majority occurring during the processing stage and the EU directive has pledged more effort in developing strategies to recover higher value components, including proteins, fibres and bioactive molecules from agricultural waste (Anon, 2019). These bioactives may have potential applications in the food and pharmaceutical sectors as antimicrobials, anti-oxidants and natural colorants (Baiano, 2014). Among the most commonly grown crops in Europe, excluding cereal crops, are olives,

rapeseeds, tomatoes, and citrus fruits, where each comprise the bulk of crop waste within each of their specific categories (Fig. 1). Within the olive industry two phase or three phase extraction of the oil is generally deployed, resulting in the formation of olive mill cake and olive mill waste water (Souilem et al., 2017). Smaller companies may also perform destoning in order to produce oils containing higher proportions of polyphenolics. The waste generated from the mills is disposed on land but this can have a serious impact requiring soil remediation (Doula et al., 2017). In contrast, rapeseed meal is often used to supplement ruminant feeds at 20-30%, providing 50% of the animals' protein requirements, although significant variations in total protein content may be caused by many different factors (Dale, 1996). Protein variability will affect the market price of this material as an animal feed, but the presence of anti-nutritional factors has affected its uptake in the wider animal nutrition sector, because glucosinolates and phytates can make it unpalatable (Dale, 1996) although some cultivars have been selectively breed which contain much lower quantities of anti-nutrients (Ghodsvalli, Khodaparast, Vosoughi, & Diosady, 2005). Citrus waste may also be used as animal feed after being dried and pelletized, although the majority is discharged to landfill due to the high cost of drying (Negro, Mancini, Ruggeri & Fino, 2016). However, new EU legislation requires that some attempt must be made to valorise the waste before landfill disposal, which could include limonene extraction and the production of biofuels. Likewise, only a small proportion of tomato waste may be used as animal feeds or as organic fertilizer, but much of it is discarded as landfill waste due to the short shelf-life of the tomatoes of less than one week (Fritsch et al., 2017). Consequently, methods are underway to develop the recovery of a range of bioactives from tomato waste.

## **2. Basis for Chemical Extraction**

65

66       The extraction of proteins from plants using alkali or acid to degrade the cellular structure is  
67 an established approach which that has led to further refinements in order to recover high yields  
68 of intact proteins from each particular plant species. After the proteins have been extracted from  
69 the cells, they are precipitated using a salting-out technique, with reagents such as ammonium  
70 sulphate or a dewatering solvent such as ethanol. It is evident from many of these studies that  
71 most of the protein present in the plant cells can be recovered using these methods, but the  
72 technical challenge is obtaining a representative diversity of all the proteins present in the plant  
73 matrix, using techniques such as gel electrophoresis under denaturing conditions to disentangle  
74 the protein chains. The tertiary structure of the proteins, which confers functional properties, is  
75 often disrupted during extraction and separation, reducing their potential applications in the food  
76 industry. Furthermore, if these proteins are in a new disordered secondary structure under  
77 neutral pH conditions, they are likely to exhibit reduced bioavailability, which negatively  
78 impacts their potential value in the animal feed or functional food sectors.

79

### 80 **3. Basis for Enzyme-assisted Extraction**

81

82       Protein extraction using a chemical approach can degrade not only the polysaccharide  
83 fraction in the plant matrix, but also the proteins being extracted, with the concomitant loss of  
84 functionality and bioavailability. Many enzymes show optimal activity ranging from weak acidic  
85 to weak alkaline conditions depending on the type of enzyme. Generally, the optimal activities  
86 of most carbohydrases occurs under low acidic conditions whereas most proteases occur under  
87 weak alkaline conditions. For example, the activity of carbohydrases on the plant cell walls of

olive pomace would result in the release of more enzymes, albeit different types such as lipooxygenase, which is the predominant form of protein in olive pomace (Montealegre et al., 2014). The majority of protein in olive seeds, rapeseed and tomato seeds are storage proteins enclosed within protein storage vacuoles (Gillespie et al., 2005; Montealegre et al., 2014; Nietzel et al., 2013), whereas a minor proportion are aleosins (protein membrane bodies) which enclose and facilitate translocation of oil across the membrane (Montealegre et al., 2014). The protein contained within seeds would be released by the selective activity of carbohydrases and pectinases in degrading plant cell walls (Rommi et al., 2014). Once released, proteases partially degrade the large molecular weight proteins into smaller soluble proteins. In contrast, most of the proteins found in tomato peels and oranges are most likely to be associated with carotenoids thereby contributing to colour formation (Vishnevetsky, Ovadis, & Vainstein, 1999).

Once the protein is released, enzymes can also limit the extent of complex formation of the extracted protein with other cell components such as carbohydrates and phytates under different physiological conditions (Serraino and Thompson, 1984; Zhan et al., 2019). However, the quantity of protein recovered using an enzyme-assisted process is often lower than with a comparable chemical process and many studies highlight this observation. Many reports described in this review use mechanical pre-treatment (e.g. sonication) alongside enzyme-assisted extraction to increase the yield. An in depth review describes many of these methods which include ultrasound, high pressure and microwave treatments (Nadar, Rao, & Rathod, 2018). However, one current problem associated with commercial application using an enzymatic approach is the high prices of enzymes (Martínez-Maqueda et al., 2013) as well as some of the problems associated with the scale up caused by lower oxygen tension, difficulty in regulating the temperature and inconsistencies with nutrients as some will sediment (Puri,

Sharma, & Barrow, 2012). Nevertheless, as future developments continue, perhaps with the ability to reuse enzymes covalently linked to nanoparticles by magnetic capture methods, it is likely that enzyme costs will decrease.

## **4. Olive Residues**

### **4.1. Olive oil production and protein rich olive constituents**

The European market has the largest production of olive oil in the world where 10.4 million tonnes of olives are processed each year, yielding 2.3 million tonnes of olive pomace and an estimated 30 million m<sup>3</sup> olive mill waste-water (Fritsch et al., 2017). Consequently, 80% of the total mass of olives harvested, results in the production of waste pomace and waste-water (Fig. 2). Furthermore, 10% of additional olive waste is generated when leaves and twigs are accidentally collected in the olive mill and in-field during pruning of the branches from trees, which is required every two years (Niaounakis and Halvadakis, 2004).

The seeds located within the centre of olive stones are one particular fraction of olive waste containing the highest concentration of proteins and oils (Rodríguez et al., 2008). Currently, the olive stones, comprising 22% of the total dry biomass, are crushed to form meal cake, which is used as animal feed. The olive seeds form 4% of the total dry biomass of olives, and the protein content comprises all of the essential amino acids, making it a suitable supplement in the human diet and as an animal feed. The stones contain 3.2% protein (Rodríguez et al., 2008), but the majority of this is composed of a woody material (Bianchi, 2003), indicating that the protein content of the seed kernels to be 18%. It would be anticipated that most of this protein would be

globulins that would be stored in specialized organelles (Montealegre et al., 2014). In addition, fresh olive leaves, accounting for 10% of the total harvest weight, (Lafka, Lazou, Sinanoglou, & Lazos, 2013) contain 7.2% crude protein in undried leaves (Aydinglu & Sargin, 2013), which are most likely to be oleosins, proteins associated with the high oil content in the leaves. However, the high concentration of polyphenols in leaves could inhibit downstream protein recovery (Romero-García et al., 2014).

#### 4.2. Chemical Processing of Olive Leaves, Pomace and Stones

The separate recovery of proteins from olive pomace and milled olive stones can be achieved using a chloroform: methanol (2:1) solvent mixture (Montealegre, Marina, & García-Ruiz, 2010). Usually, this method involves the recovery of lipids, but the association of lipids with proteins appeared selectively to assist in protein recovery. This protein isolation method was preferred to the conventional method that utilized detergents such as sodium dodecyl sulphate and 2-mercaptoethanol, because the solvents facilitated lipid extraction, which is detrimental to protein recovery. The use of two volumes of ice-cold acetone caused precipitation of protein, which co-incidentally also resulted in enzyme inactivation and maintaining polyphenol solubility. Analysis of recovered proteins from olive pomace using capillary electrophoresis indicated that seven of the major proteins were predominant throughout the different varieties of olive trees (Montealegre et al., 2012). Seeds were removed from the stones, milled under liquid nitrogen and then extracted using three separate extraction buffers, to yield a combined total of 61 globular and histone proteins (Esteve et al., 2012). In the same study, 231 proteins were recovered from olive pulp showing diverse metabolic activities including proteins that induce



allergic responses. The focus of this research was to explore the complete diversity of proteins, especially those that were in minor proportions, rather than maximise protein recovery but the results did appear to show more intense protein bands on the SDS-PAGE gel using one particular buffer. It is possible that the presence of low EDTA concentrations in this buffer caused a disruption in the enzyme activity naturally associated with the olives, thereby leading to increased protein recovery. Furthermore, the use of different buffers did not appear to influence the protein profiles obtained on the SDS-PAGE gel.

A later review by the same authors recommended the use of Tris-HCl buffer along with the detergents SDS and 2-mercaptoethanol (Montealegre et al., 2014). The presence of mercaptoethanol acts to inhibit the nascent activity of proteases naturally present in the olives. The co-extracted polyphenols were removed by repeated washing with trichloroacetone, acetone and methanol. It was reported that the protein profiles were similar to those obtained using the phenol and SDS extraction protocol, which confirms the results found in another independent study showing that different extraction buffers had little influence on the protein profiles that were obtained. The seed proteins were extracted using buffered sucrose at pH 7.5 containing salts, coordination complexes, and ascorbic acid. The protein recovered as determined using the Bradford protein assay from whole olives ranged from 0.3 to 1.2 mg/g whereas 11 mg/g was associated with stones. These results indicate that the protein concentrations associated with olives are low, but considering that the majority of the stone is devoid of protein and that only the seeds contain high quantities, it would seem prudent to develop a process to recover the more pliable seed material and leave the stone material behind.

The development of a method to extract purified proteins from olive leaves involved finely grinding the leaves and repeatedly washing them in 10% trichloroacetone in acetone to remove

polyphenols (Wang et al., 2003). The release of 2.49 mg proteins/g biomass after using phenol and SDS on the washed leaf particles, were precipitated by centrifugation after the addition of methanol to the lower phenol phase. The washing steps in trichloroacetone ensured the extracted protein was free from contaminating polyphenols and could be easily resuspended. It is evident that trichloroacetone is a useful solvent in reducing the high polyphenol content associated with olive and olive leaves.

#### 4.3. Enzyme-assisted Processing of Olive Leaves, Pomace and Stones

There is only one report describing an enzymatic approach to recover protein from olive leaves and method optimization revealed that the following conditions were necessary: 30% acetonitrile, 5% Celluclast 1.5 L, pH 5, 55°C for 15 min (Vergara-Barberán, Lerma-García, Herrero-Martínez & Simó-Alfonso, 2015). The success of extraction was evaluated by quantifying the total protein yield using the Bradford assay and the molecular weight of the proteins were examined using SDS-PAGE to reveal two different proteins, which were consistently expressed in different genetic varieties of olive trees in addition to other proteins.

An examination of different enzymes revealed that the most effective protein extraction from olive pomace was achieved using 5% lipase (Palatase 20000 L) for 15 min at 30°C with sonication, resulting in the recovery of just over 1 mg protein/ g dry biomass (Vergara-Barberán et al., 2014). Longer incubation times appeared to affect protein recovery negatively, perhaps due to the release of proteases that would be involved in protein degradation or the growth of attached microorganisms. It would appear that similar concentrations were obtained compared with a chemical non-enzyme based extraction protocol. Much higher quantities of protein were

obtained when 10 g olive pomace was treated with a protease, 80 mg Alcalase 2.4 L, in 100 ml water at pH 5 and at 50°C revealing that 0.4 g protein could be extracted, compared with 0.1 g protein extracted using the same treatment where no enzyme was used (Vioque et al., 2000).

Analysis indicated that the soluble fibre content had increased perhaps indicating that there was some side polysaccharide activity.

Protein extraction from olive stones was examined in two different studies. In one study, the protein was solubilised using milled stones in Tris-HCl buffer containing NaCl, EDTA, dithiothreitol and a protease inhibitor cocktail (Esteve et al., 2012). The proteins were purified using a ProteoMiner (BioRad), analysed on SDS-PAGE, and sequenced after trypsin digestion by mass spectrometry. This analysis resulted in the identification of 63 different proteins that were mostly globular. These results appear to be very similar to those obtained using a chemical approach. In the second study, cellulase (Celluclast 1.5L) or phospholipase (Lecitase Ultra) were most effective enzymes when using a 15 min digestion at 40°C with sonication, to obtain a protein concentration of 1 mg protein/g dry biomass as determined using the Bradford assay (Vergara-Barberán et al., 2014). It would appear that the quantity of proteins recovered using this enzyme assisted approach is ten-fold lower compared with the chemical approach.

The use of various physical treatments to increase protein recovery has been reported. These include voltage electrical discharge or ultrasonication, which was shown to significantly increase protein yield from olive kernels immersed in water at pH 7 by at least two-fold (Roselló-Soto et al., 2015). It was also found that the levels of extracted protein increased when using voltage electrical discharge at up to pH 12 or with increasing ethanol concentrations (>25%). However, polyphenols were also co-extracted with the proteins.

A comparison of the total quantities of protein associated with different components of the olive fruit revealed that leaves contained the highest levels followed by stones and finally pomace (Table 1). When each of these components were hydrolysed by different carbohydrases, a slightly higher quantity of proteins was recovered from the leaves compared with the pomace and stones. However, it would appear that a protease was more effective than the carbohydrases in recovering protein, albeit as smaller peptides.

## **5. Rapeseed Residues**

### **5.1. Rapeseed Pressing and Composition of Rapeseed Meal**

Rapeseed is an important crop for the production of vegetable oil (Canola) throughout Europe, with France, Germany, Poland and the United Kingdom the major producers. Rapeseed meal produced following the removal of the oil accounts for 80% of the waste generated from this crop (Fig. 2). Rapeseed contains a high protein content of ~34%, making it a useful supplement in animal feed (Lomascolo, Uzan-Boukhris, Sigoillot, & Fine, 2012), although a limitation is the high quantities of phenolics, which associate with the proteins to impart unusual flavours and may also act as anti-feeding agents (Alu'datt et al., 2017). Rapeseed press cake is the residual material left after defatting rapeseed by mechanical-extraction methods such as screw pressing. The application of mechanical pre-processing prepares the rapeseed material for downstream solvent extraction, which conventionally uses hexane. In addition, cold-pressing is used for the production of niche-market native rapeseed oils, with the residual material, cold-pressed press cake having an oil content of approximately 15–18%. Cold and hot pressing are performed at

60°C and 90°C, respectively, with a 10°C variation for both presses (Siger, Józefiak, Górnaś,  
2017). If an additional solvent extraction step is applied to further extract oil from the press  
cake, then rapeseed meal is obtained, which contains approximately 35–40% protein (based on  
nitrogen content using a conversion factor of 5.7 for Kjeldahl analysis) and 1–2% fat (Mosenthin  
et al., 2016). The majority of protein in rapeseed is composed of two globular storage proteins;  
mostly cruciferin and smaller quantities of napin. Cruciferins have good emulsifying properties  
whereas napins have good foaming properties that are considerably better than egg albumin  
(Rehder et al., 2017). These proteins are stored within protein bodies found throughout most  
types of cell in rapeseed (Rommi et al., 2014; Yiu, Poon, Fulcher, Altosaar, 1982).

The main limitation in using rapeseed protein for commercial applications, both in the food  
and non-food sectors, is the limited protein solubility in the press cake and press meal. One  
factor affecting solubility is the high temperature processes used during oil extraction, e.g. screw  
pressing, and downstream solvent extraction and removal, resulting in protein denaturation  
(Kemper, 2005). Despite the high levels of protein in both rapeseed cake and meal, other  
limitations for commercial applications are due to the high fibre content and the presence of  
residual anti-nutrients, in particular phytic acid, glucosinolates and phenolic compounds. The  
main technical challenge is efficient separation of the proteins from the other components such  
as carbohydrates, lignin, phenolics, and many current processes generate large volumes of  
effluent resulting in inefficient separation of the meal constituents.

## **5.2. Chemical Processing of Rapeseed and Rapeseed Meal**

The majority of studies relating to the processing of rapeseed, have focused on optimising oil extraction in order to minimise the levels of non-lipids, using different mixtures of aqueous and non-hexane solvents. Consequently, the majority of non-lipid components remain in the rapeseed meal. One report (Citeau, Regis, Carré, & Fine 2018) investigated the influence on oil extraction efficiency and rapeseed meal quality, of using ethanol and isopropanol at various water concentrations. Rapeseed oil extraction was carried out using ethanol (up to 96 wt.%), isopropanol (up to 88 wt.%), using hexane as a reference solvent. The results indicated that hydroalcoholic extraction increased meal protein content by 13% compared to hexane extraction, but the type of alcohol and proportion of water had no significance on protein yields. Therefore, replacing hexane extraction with hydroalcoholic extraction would ensure that a higher proportion of protein remains with the rapeseed meal rather than some of the protein being extracted in the hexane.

In addition, there may be environmental benefits in using isopropanol and ethanol, rather than hexane, despite the difference in polarity, which effects oil selectivity and miscibility during extraction (Breil et al., 2017). A previous study demonstrated that the extraction of de-hulled rapeseed flour with 60% ethanol or isopropanol not only increased protein concentration from 53 to 63 g / 100 g of de-oiled dry matter but also removed up to 97% of polyphenols and 99% of glucosinolates (Berot & Biffaud, 1983). Application of methanolic extraction, results in removal of phenolics, including d sinapic acid, which has potential applications in stabilising refined oils (Thiyam, Kuhlmann, Stöckmann & Schwarz, 2004).

The extraction of anti-nutritional factors from proteins is a technical challenge that needs to be addressed if material is required for use in food or animal feed applications. An early report of glucosinolate extraction from rapeseed, used aqueous and ethanolic mixtures to process

both the seed and meal, and although efficient, highlighted several disadvantages, including long (15 h) extraction periods, slow drying of the meal slurry and the dark appearance of the product (Kozłowska, Sosulski & Youngs, 1972). The ISO norm (1992) method is now the most common procedure for extracting glucosinolates from plant material, although the method requires modification to recover optimal yields from each particular plant material. A freeze-drying step, although not explicitly required in this method, prevents myrosinase mediated glucosinolate hydrolysis from occurring, which would normally occur during mechanical processing of leaf, stem or root tissues. Myrosinase, an enzyme found in Brassicaceae and compartmentalised in cells in close proximity to glucosinolates, is responsible for the hydrolysis of these glycosides during plant tissue disruption and freeze drying is used to remove water in order to prevent hydrolysis through thermal inhibition. Following freeze-drying, extraction is carried out at 75°C in 70% methanol for 10 min, in order to denature any residual myrosinase at the higher temperature. The extracted glucosinolates are then desulphated by ion exchange chromatography, separated and identified using HPLC. A simplified method for extracting glucosinolates from plant tissues, which does not require the use of a freeze drier or boiling methanol, and is therefore shorter, less hazardous and more cost effective, has been reported recently (Doheny-Adams et al., 2017). However, the use of isopropanol resulted in glucosinolates yields that were 49–73% lower in protein extracts compared with the use of other alcohols and the proportion of water present in the extraction mixture showed a correlation with glucosinolate yields.

An interesting alternative approach to reduce anti-nutritional factors such as tannins, phytate and enzyme inhibitors is the use of extrusion (Nikmaram et al., 2017). These particular compounds are high in seeds and nuts, although the effect of extrusion was dependent on

particular cultivars. A soaking pre-treatment of the biomass appears to increase the effectiveness of the extrusion process. Therefore, this method may not only result in the removal of anti-nutritional factors but also could also eliminate enzyme inhibitors leading to higher protein yields.

An alternative approach to extracting glucosinolates from rapeseed meal has been reported, which involved the chemical conversion of myrosinase to allyl isothiocyanate (Hetherington, Hoffmann, & Lindenbaum, 2018). Isothiocyanate was removed using volatile extraction that involved mild heat and negative pressure, resulting in glucosinolate levels that were 80% lower compared with the original starting material.

The extraction of cruciferin-rich protein from rapeseed meal was achieved at pH 2, using a patented procedure to collect three fractions, the rapeseed hulls, an insoluble protein fraction and a soluble protein fraction through a process of decanting and membrane filtration. The rapeseed meal contained an initial protein content of 27% and 22 µg/ kg glucosinolates, but following extraction the protein content in the insoluble and soluble fractions increased to 42% and to 58%, with a reduction in glucosinolate concentration to 1 µg/ kg and 3 µg/ kg, respectively (Rehder et al., 2017).

Only one pilot scale protein extraction study has been described, that involved a two-stage aqueous washing extraction of dehulled rapeseed meal (Fauduet et al., 1995), using 15 kg of meal and 90 kg of deionised water, which was stirred for 30 min at 18°C and filtered, leaving material with a lower glucosinolate content of ~7%. Higher quantities of glucosinolates were removed with increasing temperatures. Limitations in scaling up this process in order to upgrade the rapeseed meal were lower yields with increasing biomass used and increasing incubation periods, but modifications were proposed, including use of a countercurrent extraction system.



The use of physical pre-processing to assist with recovery of protein enriched fractions from rapeseed was investigated by Laguna et al (2018). The impact of particle size on the efficiency of dry fractionation processes, including the use ultrafine milling and electrostatic sorting/ turbo separation was reported. The milling step was designed to release the rapeseed components from the cellular matrix, whilst the electrostatic separation was used to fractionate the protein without any loss of functionality. It was noted that although high purity protein fractions were obtained using this approach, four additional recycling steps were necessary in order to increase the final yield to 30%.

### **5.3. Enzyme-assisted Processing of Rapeseed Meal**

Sari, Mulder, Sanders, & Bruins, (2015) reviewed details of different combined physical pre-treatment and enzymatic fractionation processes used to separate proteins for a range of biomass feedstocks, including rapeseed. This review highlighted the use of both proteases and carbohydrases that were applied to assist in protein extraction, with proteases aiding the fractionation process through proteolysis, while carbohydrases assisted by degrading component parts of the cell wall. It was noted that conventional alkaline extraction can be improved by protease addition, due to the reduction in protein size through proteolysis which facilitates easier extraction. In addition, the use of proteases can also be used to enable lower processing pH, thus avoiding the severe conditions that denature protein, with a resultant loss of potential functionality. The use of proteases was used in one study to improve the release of oil from dehulled ground rapeseeds by comparing five different proteases and it was reported that Alcalase 2.4L was the most effective (Meng et al., 2018). The rapeseeds were boiled in water for

15 min, extracted under alkaline conditions at pH 9, treated with 1.5% Alcalase 2.4L at pH 8.5, 55°C for 4 h and then inactivated at 90°C. The extracted proteins after this treatment had become structurally disordered with a reduction in the proportion of  $\alpha$ -helix chains by 30%.

In a separate study, protein extraction from milled rapeseed, was examined using a variety of different Protex proteases. Higher reported protein extraction yields of 60-80% were obtained, using alkaline proteases rather than acidic proteases at 5% loading and pH ranging from 9.5-11, at 60°C and for 3 h (Sari, Bruins, & Sanders, 2013). Another study investigated protein extraction from pre-pressed (PPM) and cold-pressed rapeseed meal (CPM) under different parameters, that included variations in the solid to liquid ratio, extraction time, temperature, pH value, the number of extraction cycles and the employment of a protease- Protease A-01 (Subtilisin, EC 3.4.21.62) (Fetzer et al., 2018). The highest protein yields achieved were 60.6% from PPM and 59.5% from CPM using protease activity in the presence of strong alkaline conditions, pH 11-12, during a single step process. In a triple washing-step process, 78.3% and 80.7% was recovered from PPM and CPM, respectively.

In another report highlighting the use of proteases to increase protein recovery, casein was immobilized onto the surface of magnetic nanoparticles, resulting in the hydrolysis of 47% of the protein into amino acid and oligopeptides (Jin et al., 2010). However, the hydrolysis of rapeseed meal using these nanoparticles indicated that only 10% of the total amount present was hydrolysed, although this occurred at similar rate to the free enzyme. The advantage of using this system was that the protease retained activity up to 60 days at 4°C and could be easily recycled.

In another variation, the protease was used after the proteins had been recovered by alkaline extraction, with the aim of increasing the purity of the extracted protein. High purity protein (92%) was obtained from ground rapeseed meal after washing with ethanol, extracting in an

alkaline NaCl solution and collecting the fraction <10k Da by ultrafiltration (Zinchenko et al., 2018). The proteins were successfully degraded into amino acids and oligopeptides when the extracted proteins were incubated with protosubtilin at a ratio of 20:1.

The use of carbohydrases to degrade cell wall components that retain the protein, rather than extracting the proteins directly from the plant substrate is an alternative approach. The highest yield of proteins of 50 mg/ g meal was obtained when phenolic acids and proteins were recovered in a sequential reaction. This involved the addition of sodium hydroxide and methanol to form phenolic acid esters, which were evaporated and the protein was extracted under alkaline conditions followed by precipitation under acidic conditions (Li & Guo, 2017). Cellic Ctec3 was used at a later stage to purify the extracted protein when incubated at 50°C. The recovery from rapeseed meal of sinapine (the ester form of sinapic acid – a dominant phenolic acid) was 7 mg/ g and of protein was 0.5 g/ g. This protein had an enrichment content of 77%. In another study, the effect of carbohydrases on rapeseeds were determined by fluorescence microscopy when stained with Calcofluor to view remaining glucans and with Acid Fuchsin to view the protein distribution (Rommi et al., 2014). In addition, pectins were examined by microscopy after staining with Ruthenium red. Pectinex Ultra SP-1 showed the highest activity compared with Celluclast 1.5 L and Depol 740L, resulting in the complete disintegration of the cell walls, which contained the protein and the release of protein bound to pectins. Higher levels of protein were recovered from the dehulled seeds compared to the intact material, and SDS-PAGE revealed that napins were present at higher concentrations in protein extracts from the dehulled seeds. High yields of proteins can be recovered from cold pressed rapeseed meal using alkaline methods but these require large volumes of water and it was found using Pectinex Ultra SP-L resulted in higher yields under low moisture conditions (Rommi et al., 2015). It was also determined that

particle size had no effect on the protein recovery but enzyme activity increased protein recovery by 29-42% when extracted at 20% solid content.

There are a few reports citing the combined use of carbohydrases and proteinases to improve the extraction of protein of oil from rapeseeds. In one such study, a multi-enzyme approach using pectinase/ cellulase/ betaglucanase, Alcalase 2.4L, at pH 5-10, and a temperature range of 48-60°C, yielded 41-67% protein depending on the hydrolysis time (Zhang, Wang, & Xu, 2007a). Sari, Mulder, Sanders, & Bruins (2015) concluded that the application of carbohydrases, as part of the hydrolysis process, does not appear to result in increased yields of extracted protein, although their use may have a positive impact on protein extraction in a different way. Their capability to degrade the cell wall can be used to release components that otherwise buffer the reaction mixture, which would result in lower alkali consumption during subsequent protein extraction and a reduction in process costs. However, another study reported the sequential use of carbohydrases and proteases to successfully isolate a protein fraction from rapeseed. This study examined the effect of 2.5% pectinase, cellulase and  $\beta$ -glucanase at the optimised ratio of 4:1:1 on wet, milled dehulled rapeseeds for 4 h (Zhang, Wang, & Xu, 2007b). This was followed with alkaline extraction at 60°C for 1 h and 200 rpm, and then protease treatment, Alcalase 2.4L, by adjusting to pH 9 at 60°C and 50 rpm at enzyme concentrations ranging from 0.5% to 1.5% and solid to liquid ratio ranging from 1:3 to 1:8. It was found that the optimum conditions were 1.25-1.5% Alcalase 2.4L at 50°C for 3 h to recover about 80% of the protein with a molecular weight of less than 1500. The proteins were analysed after centrifugation by collecting the liquid fraction between the remaining seed pellet and extracted oil forming an upper surface layer.

Another approach that overcomes the anti-nutritional factors associated with rapeseed meal involves the use of 0.8 U/g of phytase at 55°C, pH 5, which reduced phytic acid content by 25%

phytase (Rodrigues, Carvalho, & Rocha, 2017). The protein yield obtained, as determined by Bradford assay, was optimum at 75°C, under alkaline conditions at pH 12.5, and then re-precipitating at pH 4. The phytic acid contents of rapeseed meal, defatted rapeseed meal and protein extract were 14 g/ kg, 10 g/ kg and 1 g/ kg, respectively.

A much higher quantity of protein was associated with rapeseed compared with other crops, especially with the cold pressed rapeseed meal, which contains lower quantity of oil and is pressed under low temperatures that would limit protein denaturation (Table 1). Studies where carbohydrases were deployed do not appear to describe the protein yields, except the study by Li & Guo (2017), where the enzyme was used after alkaline extraction in order to remove co-extracted carbohydrates. It would appear that proteases were very effective in recovering the majority of protein from rapeseed meal.

## **6. Tomato Residues**

### **6.1. Production and Tomato Constituents**

Tomato farming occurs throughout Europe, generating about 17% of waste. This is lower compared with the levels of waste generated from the production of olives and rapeseed (Fig. 2), although larger quantities of waste accumulate in Italy and Spain reflecting the larger extent of tomato farming in these countries. The dietary fibre associated with tomato waste is the most important constituent, forming 80% of the biomass, which is recovered using a patented process where the peels are ground after being separated from the seeds and then dried (Herrera, Sánchez-Mata, & Cámara, 2010). The proportions of total dry biomass and seeds in tomato

waste account for 15.8% and 3.5%, respectively (Zuorro, Lavecchia, Medici, & Piga, 2014). Most of the protein is associated with the tomato seeds at 35-40% and this protein contains most of the essential amino acids, except tryptophan that was present at lower amounts (Sarkar & Kaul, 2014; Zuorro, Lavecchia, Medici, & Piga, 2014). Globulins, storage proteins, comprise 70% of the total proteins in tomatoes (Sogi, Arora, Garg, Bawa, 2002a), which are most probably associated with the seeds. Another study reported that while tomato seeds have a quite high protein content, the predominant amino acids present were those with lower levels of digestibility e.g. arginine and asparagine (Persia, Parsons, Schang, & Azcona, 2003). Feeding experiments to chicks revealed that tomato seeds could substitute soyabean meal, although the weight gain of the chicks was lower, but higher compared with using a non-nitrogen feed. The same study also revealed that the tomato seed composition showed disparity between different cultivars but did show consistency within different samples collected from the same farm (Persia, Parsons, Schang, & Azcona, 2003). The tomato seeds have quite a high content of anti-nutritional factors in the form of phytate (26 µg/g) and trypsin inhibitors (12.5 U/mg), but these inhibitors can readily be reduced >80% with the removal of the bran from the seed to recover protein (Sarkar & Kaul, 2014).

## 6.2. Chemical Processing of Tomato Seeds

The majority of studies have focused on protein recovery from tomato seeds and a number of similar methods have been described using alkaline extraction. In one of the first described methods using standard alkaline processing, proteins were extracted from different fractions of tomato waste that was solubilised at pH 8, pressed and then the pH was sequentially reduced to

pH 4.8, pH 4 and finally pH 3.5, in order to collect different protein concentrates as the proteins precipitated (Kramer & Kwee, 1977). The proportion of soluble protein increased from 35% to 56% as the pH changed from pH 4.5 to pH 3.5. A further development in another study, examined the purity level of proteins recovered when the proteins were precipitated at pH 3.9 (Liadakis, Tzia, Oreopoulou, & Thomopoulos, 1995). In this study, proteins were extracted from tomato seed meal using water at a liquid to solid ratio of 30:1 at 50°C and pH 11.5 for 20 min. The solids were removed using centrifugation, the pH of the supernatant was adjusted to 3.9, and the precipitated proteins were collected by centrifugation. The final product after vacuum drying contained 72% protein. In another shorter method using weak alkaline conditions, the tomato seeds were separated from the skins using sedimentation which were then subjected to sodium hydroxide treatment for 5 min, centrifuged and the supernatant was adjusted to pH 7.5 (Savadkoohi & Farahnaky, 2012). The tomato seed protein was centrifuged and structural chemical analysis revealed that the globular protein exhibited weak gelling properties. In another method, the use of different solvents for extraction was investigated after using hexane to remove oil from the tomato seed meal and recovering the proteins using alkaline conditions with 1.2% sodium hydroxide (Sogi, Arora, Garg, & Bawa, 2002a). Extraction with water, ethanol or acetic acid resulted in the recovery of different molecular weight proteins under each of the extraction strategies ranging from 67-310 kDa.

The emulsifying properties of the extracted proteins were evaluated to determine their potential functionality for applications as food ingredients. In one of these studies, the emulsion properties were evaluated against water and peanut oil, after the proteins were extracted from sedimented, hammer-milled tomato seeds, using 1% NaOH at ambient temperature for 10 min (Sogi, Garg, & Bawa, 2002b). The protein concentrates and isolates from the seed meal showed

improved emulsifying properties and much lower absorption of water compared with peanut oil. In another study, the emulsifying properties of the proteins extracted from tomato seed protein were found to be stable in high sodium chloride concentrations and thermally stable to 80°C, whereupon the proteins aggregated and were stable within the pH range 6-8 (Sarkar, Kamaruddin, Bentley, & Wang, 2016). The proteins were extracted from hammer-milled seeds, soaked for 1 h in 1 M sodium chloride at 50°C, adjusting to pH 8 with sodium hydroxide, centrifuging to remove non-proteins and then readjusting the pH to 3.5 and centrifuging the proteins.

Tomato waste was pulped and the seeds were separated from the peels by sedimentation (Sarkar & Kaul, 2014). The seeds were then hexane extracted to lower the fat content and the seed protein was extracted using 1 M NaCl, which was maintained at pH 8 for 1 h at 50°C. The remaining biomass was centrifuged and the extracted protein was precipitated with the addition of HCl to form a protein isolate of 92%.

### **6.3. Enzyme-assisted Processing of Tomatoes and Tomato Seeds**

The ripening of tomatoes may provide some guidance as to the type of enzymes that could be involved in softening the fruit. Tomassen, Barrett, van der Valk, & Woltering (2007) described an activating enzyme that was found to modify a pectin-degrading enzyme, polygalacturonase, into an active isoenzyme state. The protein was recovered from ripe tomatoes after gentle heating of the extracted enzyme to separate the combined enzymes.

Only one study has been reported involving the enzyme mediated extraction of proteins from either whole tomatoes or a specific component of tomatoes, although the use of enzymes have



been successfully employed in the recovery of other carotenoids and lycopene. The extraction of umami acids from defatted tomato seed meal was achieved using papain and it was found that at pH 3, high enzyme activity and a long incubation period of 5 h resulted in extract containing 86% of protein (Zhang et al., 2015). The tomato seeds were milled and the resultant particle size was 0.43- 0.85 mm. About 50% of the protein was extracted from this material, but decreasing the particle size further to <0.25 mm resulted in an increase in yield to 90%.

The tomato seeds appear to contain a high protein content although there is some variation depending on the cultivar being assessed (Table 1). It would appear that all of the protein was recovered from the seeds when a protease was used.

## **7. Citrus Residues**

### **7.1. Production and Citrus Constituents**

The cultivation of citrus fruits, comprising mostly of oranges, tangerines, lemons, limes and grapefruit, occurs only in southern Europe. It is estimated that 3.2 million tonnes are deemed unsuitable and processing of these fruits generates a significant proportion of waste products after juicing, which is composed of the peel, pulp, rag and seeds at 1.6 million tonnes (Fig. 2). The waste could be useful in bioethanol production especially considering the high cellulosic content (cellulose and hemicelluloses), with a particularly low lignin content that can vary from being undetectable to 7.5% in orange peels (Mamma & Christakopoulos, 2014). Citrus waste has a low protein content, between 6.6-9.1% in both the peels and pulp, and proposals have suggested increasing the protein content by using microbial fermentation to form single cell protein.

## 7.2. Chemical Processing of Citrus

An extrusion process was developed whereby equal proportions of whey proteins and citrus pectins were covalently linked to produce compounds that showed improved emulsifying properties at 120-140°C (Koch, Emin, & Schuchmann, 2017). It was determined that during heat treatment of these whey proteins, their solubility decreased, whereas viscosity increased due to the increase in molecular weight of the protein-polysaccharide conjugants and then gradually decreased due the degradation of polysaccharides. The emulsifying properties as determined by smaller droplet sizes improved after 2 min of extrusion at 140°C , but longer incubation times did not result in any further improvements. The soluble protein content of citrus juices originating from the flavedo (the outer orange coloured peel) showed a significant decrease with increasing temperatures up to 100°C, resulting in insoluble precipitates causing increased cloudiness of the fruit juice (Shomer, 1991). However, protein insolubility was also influenced by enzymatic degradation of pectins at pH 4.5 into neutral sugars and galacturonic acid. It was found that the protein coagulants particularly contained arabinose and galacturonic acid.

## 7.3. Enzyme-assisted Processing of Citrus Pulp and Peel

An enzymatic extraction of orange peel using a buffer containing Celluclast 1.5L from different cultivars resulted in the recovery of 5.45 mg proteins/ g peel, as determined by the Bradford assay (Vergara-Barberán et al., 2017). Protein separation was achieved on the basis of molecular weight, using capillary gel electrophoresis and assigned to particular roles based on

previous published research. Many of the 14 common proteins were either allergens or enzymes, while other proteins were unique to particular cultivars.

Only one study investigated protein extraction from citrus pulp, which revealed that Palatase 20,000 L was more effective, albeit at lower yields, in recovering 1.7 mg protein/ g pulp (Vergara-Barberán et al., 2017). The results obtained were similar to those found using citrus peels where eight of the proteins were common within citrus fruits while other proteins were unique to particular cultivars.

The quantity of protein associated with the citrus peels is comparable to the quantity that can be recovered from the olive leaves (Table 1). It would be expected that carbohydrases would be effective in releasing protein from the peels, considering the high cellulose content of the peels. However, only a small proportion of the protein was recovered and no study has appeared to determine whether higher quantity of protein could be recovered using a protease.

## **8. Conclusions**

It is apparent that the optimum recovery of proteins from each of these crop residues using the chemical methods rely on organic solvents, alkalis or acids, which may be environmentally hazardous. In contrast, there are many studies showing the development of methods to incorporate an enzymatic approach to recovering proteins from different components of crop residues. At this stage, it would appear that proteases operating under low alkaline conditions are more effective than carbohydrases in recovering plant protein, although the hydrolytic activity of proteases results in the generation of low molecular weight peptides. It is most likely that intact functional proteins would be recovered using carbohydrases and that these intact

proteins would be useful in human and animal feed, because of the potential to impart additional functionality through partial hydrolysis. Nevertheless, it is clear that methods are being developed for the recovery of protein using enzymatic assisted extraction and this approach is being investigated using a wide range of agri-food residues.

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## List of Figures

Fig. 1 The percentages of the total quantities of crops and different categories of crops grown in Europe. Compiled using data (Union européenne, 2018). The total production of all crops, vegetables, fruits and oilseeds amounts to 988.8 mT, 64.8 mT, 32.6 mT and 35.0 mT, respectively.

Fig. 2 The percentages of crop waste from processing of 16.3 mT of tomatoes (Scherhauser et al., 2018), 6.2 mT of oranges (Ferreira-Leitao et al., 2010; Rezzadori et al., 2012), and 10.3 mT of olives and 21.9 mT of rapeseed (Searle and Malins, 2013). Most of the olives and rapeseeds remain once the oils have been extracted, while the waste from tomatoes is seemingly low although 82% of the total weight is composed of moisture and the squeezing of oranges for juice leaves behind peel, pith and seeds.

Table 1 The determination of total protein associated with each of the crops (no enzyme) and protein extracted using different enzymes. A chemical approach was used when the entry is described as none in the column labelled as enzyme.

Table 1

<b>Sample</b>	<b>Enzyme</b>	<b>Protein</b>	<b>Reference</b>
Olive leaves	None	7.2%	Aydinglu & Sargin, 2013
Olive pomace	None	0.1-1.2%	Montealegre et al., 2014
Olive stones	None	3.2%	Rodríguezb et al., 2008
Olive leaves	Celluclast 1.5L	0.2-0.7%	Vergara-Barberán, Lerma-García, Herrero-Martínez & Simó-Alfonso, 2015
Olive pomace	Palatase 20000	0.1%	Vergara-Barberán et al., 2014
Olive pomace	Alcalase	4%	Vioque et al., 2000
Olive stones	Celluclast 1.5L	0.1%	Vergara-Barberán et al., 2014
Rapeseed	None	33.9%	Lomascolo, Uzan-Boukhris, Sigoillot, & Fine, 2012
Rapeseed meal	None	35-40%	Mosenthin et al., 2016
CPRM	None	40.6%	Fetzer et al., 2018
PPRM	None	34.4%	Fetzer et al., 2018
Rapeseed	Protex proteases	15.8-21.0%	Sari, Bruins, & Sanders, 2013
CPRM	Protease A-01	24.2%	Fetzer et al., 2018
PPRM	Protease A-01	20.8%	Fetzer et al., 2018
Rapeseed meal	Cellic Ctec3	50%	Li & Guo, 2017
Tomato seeds	None	35-58.7%	Sarkar & Kaul, 2014; Zuorro, Lavecchia, Medici, & Piga, 2014; Zhang et al., 2015
Tomato seeds	Papain	50.3%	Zhang et al., 2015
Citrus peel and pulp	None	6.6-9.1%	Mamma & Christakopoulos, 2014
Citrus peel	Celluclast 1.5L	0.5%	Vergara-Barberán et al., 2017

Abbreviations: CPRM cold pressed rapeseed meal; PPRM pre-pressed rapeseed meal